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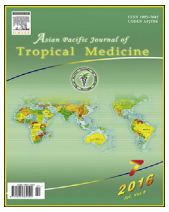

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# Ultrasonic diagnosis of patients with clonorchiasis and preliminary study of pathogenic mechanism

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ABSTRACT

**Objective:** To discuss the liver function damage mechanism of patients with clonorchiasis by analyzing the ultrasound characteristics, liver function, change of the serum inflammatory factors and cell apoptosis factors.

**Methods:** Color Doppler ultrasound technique was adopted to detect the portal vein and blood flow change of patients with clonorchiasis; ELISA was used to determine the level of different serum inflammatory factors. The levels of serum total bilirubin, serum albumin and glutamic-pyruvic transaminase were detected by automatic biochemical analyzer. Western blot was used to determine the expression of proteins relevant to apoptosis.

**Results:** Compared with the health control group, the trunk diameter of portal vein and the thickness of spleen, as well as the hepatic artery pulsation index of clonorchiasis patients increased obviously, the mean blood flow velocity of portal vein ($P < 0.05$ or $P < 0.01$) decreased. The content of total bilirubin and transaminase in plasma increased significantly, but albumin decreased ($P < 0.05$). Levels of TNF-α, IL-6 and IFN-γ increased remarkably, and the level of every factor was significantly different among patients with Child-Pugh , Child-Pugh II and Child-Pugh III classification of liver function ($P < 0.05$ or $P < 0.01$). With the exacerbation of liver dysfunction, levels of TNF-α, IL-6 and IFN-γ gradually increased ($P < 0.05$). Compared with the healthy control group, the expression quantity of apoptosis protein Fas, FasL, Bax and Caspase-3 increased significantly ($P < 0.05$ or $P < 0.01$), but Bcl-2 decreased ($P < 0.05$).

**Conclusions:** Changes of ultrasonic characteristics and liver dysfunction, caused by liver fluke infection, may be related to that both inflammatory response and apoptosis response have participated in the pathogenic process and liver damage course of clonorchiasis.

## 1. Introduction

With the improvement of living standards and diversification of diet, the incidence of the clonorchiasis is increasing year by year. Clonorchiasis is a kind of food-borne parasitic disease which is caused by the parasitism of *Clonorchis sinensis* on the human intra-hepatic bile duct. And clonorchiasis was ever ranked as the first kind of risk factors by the World Health Organization [1]. At present, common examinations for clonorchiasis diagnosis include hematology, immunology, parasitism, ultrasound and CT examination [2]. Among which, the ultrasound diagnosis has the advantages of noninvasive, shortcut and low cost. Color Doppler ultrasound used for diagnosing clonorchiasis is based on the ultrasonogram of patients' pathological change, which has provided the basis for the early diagnosis of clonorchiasis.

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In the parasitic infection, the role of cytokines has attracted more and more attention. Adult liver fluke damages the liver tissue and leads to the change of the patient's cellular immunity and humoral immunity function [3]. It has reported that the clonorchiasis patient may produce specific immune response [4] which is often accompanied by complications like jaundice, cholangitis, cholelithiasis, liver cirrhosis, biliary duct cancer, liver cell cancer and other liver and gall diseases [5–8]. This seriously threatens the life of patients. The cytokines TNF-α, IL-6 and IFN-γ are important mediums of immunoreaction in various infectious diseases. Their functions in the pathogenesis and pathological changes of clonorchiasis, and the relations with liver function injury remain unclear. This study will preliminarily explore their clinical significance in the clonorchiasis. In addition, when the liver cells suffer damage, the cytokines FasL/Fas, TNF-α, TGF-β1 and INF-γ are activated to induce apoptosis by combining with the receptor from the target cells [9]. Studies have suggested that the pathogenesis of many kinds of liver damage, including hepatic failure, viral hepatitis, cirrhosis, autoimmune liver disease and liver tumors are closely related to the apoptosis [10]. To study the liver cell apoptosis of clonorchiasis patients is of important significance on the analysis of the liver damage mechanism and the treatment of related diseases.

## 2. Material and methods

### 2.1. General materials

Sixty-eight clonorchiasis patients treated in the First Affiliated Hospital of Zhengzhou University from March 2013 to February 2015 were collected, including 47 males and 21 females, age among 19–69 years old, with average age as (45.7 ± 3.1) years old. Among which, 41 cases were confirmed as positive by liver fluke enzyme mark, 20 cases were confirmed by checking eggs in stool, 7 cases were confirmed by checking eggs in duodenal fluid using gastroscope. The healthy control group had 25 cases, including 15 males and 10 females, aged 21 to 70, with average age as (56.5 ± 1.8). All the subjects were ruled out other parasitic infections and anaphylactic disease, as well as intemperance, autoimmunity disease, viral hepatitis. And they volunteered to participate and signed the informed consent form. In additional, five human liver tissue species from clonorchiasis patients and five healthy liver tissue species conserved in the hospital were taken respectively to do control experiment.

### 2.2. Ultrasonic diagnosis

Patients were maintained at supine position. The patients' liver parenchyma, biliary system and the thickness of the spleen were observed by the Color Doppler Ultrasonic Diagnosis Apparatus (Phillips Envisor, 3.5 MHz transducer frequency). Then the portal trunk inner diameter, frequency spectrum and hepatic artery pulsation indexes were measured.

### 2.3. Measurement of the liver function

The serum total billirubin (TBIL), serum albumin (ALB) and glutamic-pyruvic transaminase (ALT) content of the subjects were detected by the fully automatic biochemical analyzer. After obtaining the data, the patients were classified according to the Child-Pugh classification standard [11]. Class : TBIL <34 μmol/L, ALB >35 g/L, ALT <40 IU/L; Class II: TBIL (34–51) μmol/L, ALT (40–55) IU/L; Class III: TBIL >51 μmol/L, ALB <28 g/L, ALT >55 IU/L.

### 2.4. Determination of serum inflammatory factors level

All the subjects were collected 6 mL elbow venous blood in the morning with an empty belly. Blood samples were sitting for 4 h without anticoagulation. Then, they were centrifuged for 15 min at 2000 rpm to separate the serum and saved under −20 °C for standby application. Serum TNF-α, IL-6 and IFN-γ levels were detected by double antibody sandwich ABC-ELISA method. Human TNF-α, IL-6 and IFN-γ kits were bought from Beijing Jingmei Biological Engineering Company. The measuring instrument was ELx800 fully automatic microplate reader (BioTek, USA). The detailed detection procedures were operated according to the instruction of the kits.

### 2.5. Apoptosis-related proteins expression in the liver tissue

The liver tissue samples saved in liquid nitrogen were taken out. Fifty mg liver tissue was fetched and grinded. Five hundred μL tissue lysis buffer was added. The sample was ice-bathed for 1 h at 4 °C, and then centrifuged for 30 min at 13000 rpm. The supernate was collected and determined its total protein content with Bradford method. The target protein was separated from the extracted total protein by SDS-PAGE and then was transferred onto the PVDF membrane. It was sealed with TBST liquid containing 50 g/L skim milk under room temperature. Rabbit anti-human polyclonal antibody Fas (1: 200)/FasL (1: 200)/Bcl-2 (1: 500)/Bax (1: 200)/Caspase-3 (1: 100)/β-actin (1: 500) was added and incubated at 4 °C. After rinsing, the goat anti-rabbit IgG-HPR antibody was added and hybridly incubated, then and rinsed. All the antibodies were bought from Wuhan Boster Biological Engineering Company. Western blot bands were scanned and the gray level was analyzed by Quantity One software. The ratio of the target protein gray level to that of β-actin was regarded as the relative expression level.

## 3. Results

### 3.1. Ultrasonic diagnostic features of clonorchiasis patients

According to the analysis of the ultrasonoscopy, it was found that in the clonorchiasis patients, the local vessel expansion appeared in intrahepatic bile duct whose wall became thickened. The echo increased and the liver parenchyma changed. From the results of detecting related indexes by ultrasound, the internal diameter of portal vein, thickness of spleen and hepatic artery pulsation index in clonorchiasis patients had increased obviously compared with the normal group, but mean blood flow velocity of portal vein decreased, and the differences were of statistical significance ($P < 0.05$ or $P < 0.01$).

Comparison of the ultrasonic testing indicators was as follows. In Normal group ($n = 25$) and Clonorchiasis group ($n = 68$): Internal diameter of the portal vein was (8.5 ± 1.9) mm and (14.7 ± 2.8), respectively; Thickness of spleen was

(30.6 ± 3.4) mm and (44.1 ± 5.0), respectively; Mean blood flow velocity of portal vein was (17.3 ± 0.6) and (7.4 ± 3.8), respectively; Hepatic artery pulsation index was (1.38 ± 0.25) and (1.56 ± 0.22), respectively.

### 3.2. Blood plasma liver function parameters of clonorchiasis patients and the Child-Pugh classification

Determination of the liver function index was as follows. Normal group: TBIL was (6.29 ± 3.12) μmol/L, ALB was (40.15 ± 6.88) g/L, ALT was (19.25 ± 8.97) IU/L; Clonorchiasis group: TBIL was (14.97 ± 8.94) μmol/L, ALB was (27.87 ± 3.26) g/L, ALT was (41.32 ± 7.74) IU/L.

Results of blood plasma liver function detected by fully automatic biochemical analyzer showed that, the TBIL and ALT level of the clonorchiasis patients increased obviously compared with the normal group, but the ALB level decreased greatly. The differences were of statistical significance ($P < 0.05$). According to the Child-Pugh evaluation standard, among the 68 clonorchiasis patients, 23 cases were Grade , 26 cases were Grade II and 19 cases were Grade III.

### 3.3. Determination of serum inflammatory factors TNF-α, IL-6 and IFN-γ levels in clonorchiasis patients

Detection of serum inflammatory level was as follows. Normal group ($n = 25$): TNF-α was (15.12 ± 3.18), IL-6 was (18.23 ± 2.87), IFN-γ was (1.29 ± 0.31); Normal group ($n = 68$): TNF-α was (29.47 ± 15.22), IL-6 was (23.07 ± 1.99), IFN-γ was (1.57 ± 0.44).

The detected results indicated that the level of serum inflammatory factors TNF-α, IL-6 and IFN-γ levels in clonorchiasis patients were significantly higher than that in the normal group ($P < 0.05$).

### 3.4. Correlation analysis of the liver function classification and the serum inflammatory level in clonorchiasis patients

By the analysis, we found that the serum TNF-α, IL-6 and IFN-γ levels were different in clonorchiasis patients with different Child-Pugh classification ($P < 0.05$ or $P < 0.01$). In addition, with the increasing of the liver function damage extent, levels of TNF-α, IL-6 and IFN-γ increased gradually.

### 3.5. Detection of the apoptosis-related protein expression

Compared with the normal group, the expression quantity of liver cell apoptosis protein Fas, FasL, Bax and Caspase-3 increased obviously, but the Bcl-2 expression down-regulated ($P < 0.05$). It is indicated that the degree of cell apoptosis in clonorchiasis patients was greatly higher than that in healthy subjects.

## 4. Discussion

In the epidemic region of *C. sinensis*, the related diseases caused by *C. sinensis* infection have been regarded as a focus of public health problem, which is closely related to life style and living surroundings. After infected by *C. sinensis*, phenomenons like bile duct wall thickening, epithelial cells degeneration and falling off, cholestasis and haemodynamics changes would happen to the host because of the mechanical irritation and block. This will lead to obstructive jaundice [5] or billary calculi [6], even cancer in severe cases [12].

In the study, determining the echo change of the intrahepatic bile duct with Color Doppler Ultrasound and observing the liver parenchyma, portal vein and the hepatic artery situation can not only qualitative diagnose the clonorchiasis disease, but also provide favorable opportunity for the accurate treatment means. In addition, the method is easily to operate with advantages of high repeatability and low requirements for ultrasonic instruments, which is beneficial for popularization and utilization. What's more, the ultrasonic testing is easier to be accepted by patients than the laboratory examination. Therefore, the ultrasonic diagnosis is the primary diagnostic method for suspected cases of clonorchiasis.

During the infection process of parasite, the immunological balance exists between the host and the parasite [13]. Parasitic infection will activate Th2, and the produced cytokines IL-6 can inhibit the immunocompetence of macrophage activated by IFN, which will aggravate the damage to the body [14]. As the main cytokines, IL-6 and TNF-α can mediate autoimmune response against liver cells, further aggravating the damage of the liver cells. TNF-α is produced by the activated macrophage and it can promote the proliferation of the B cells in the immunoreactions, increase the vasopermeability and induce the neutrophile granulocyte recruited to the infection site [15]. But the excessive release of TNF-α will bring obvious liver tissue damage. IFN-γ can adjust the immunologic function *in vivo* and maintain the stability and defensiveness of cell function [16]. In the serum of clonorchiasis patients, the level of cytokines TNF-α, IL-6 and IFN-γ increased significantly, which suggested that immunoreaction and inflammatory response had participated in the pathogenic process of clonorchiasis disease.

*C. sinensis* infection leads to the changes of cell mRNA and caspase-3 in the apoptotic pathway mediated by Fas/FasL. The following programmed cell death will ultimately destroy the liver anatomical and functional mechanism [17]. Whether the apoptosis of the liver cell is caused by the constantly stimulation of the worm or the effect of inflammatory cell produced by the infection cannot be determined yet. The unknown factors produced by the excretory-secretory antigen of the worm will directly or indirectly produce some kind of morbid substance, which may be the apoptosis signal to stimulate the liver cells apoptosis, leading to liver damage such as the change of the serum enzymes. It is obvious from the experimental results that the apoptosis protein expression quantity of Fas, FasL, Bax and Caspase-3 in liver cells of clonorchiasis had increased significantly, but Bcl-2 decreased. It is suggested that the process of the liver fluke infection leading to liver damage is accompanied by the cell apoptosis response. Studies believe that the protection of the liver can be achieved by the inhibiting cell apoptosis. While inhibiting the liver cell apoptosis mediated by TNF-α can reduce the degree of liver damage [18].

## Conflict of interest statement

We declare that we have no conflict of interest.

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